

Improved film properties of radiation-treated medium-chain-length poly(hydroxyalkanoates)

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Medium-chain-length poly(hydroxyalkanoates) (*mcl*-PHAs) were synthesized from coconut oil (PHA-C), tallow (PHA-T), and soybean oil (PHA-S) by bacterial fermentation using *Pseudomonas resinovorans* as the producer strain. Films were solution-cast and subjected to 50 kGy of γ -irradiation. This resulted in crosslink formation based on the number of olefinic groups present in the polymer side-chains. In each case, radiation improved the tensile strength (104% and 63%), percent elongation (49% and 13%), and Young's modulus (30% and 76%) of PHA-C and PHA-T films, respectively. The greatest effect was on PHA-S, which was converted from an amorphous, liquid-like material to a solid elastomeric film.

Keywords: γ -irradiation, crosslinked poly(hydroxyalkanoates), film properties, *Pseudomonas resinovorans*, triacylglycerols.

Introduction

Poly(hydroxyalkanoates) (PHA) are chiral polyesters that are produced intracellularly by a number of bacteria as carbon and energy storage reserves. For example, *Pseudomonas resinovorans* produces medium-chain-length (*mcl*) PHA from animal fats and vegetable oils (Cromwick *et al.*, 1996; Ashby and Foglia, 1998). These polymers are composed of 3-hydroxy acid monomer units ranging in length from C4 (3-hydroxybutyrate) to C14 (3-hydroxytetradecanoate). Each *mcl*-PHA contains a unique number of side-chain olefinic groups based on the triacylglycerol (TAG) fatty acid composition and the specificity of the polymerization enzymes. An increased concentration of unsaturated side-chains results in polymers with properties ranging from elastomeric to adhesive-like (tacky). As such, the films produced from these polymers are either weak or non-coherent unless the film matrix is modified.

One way to strengthen a polymer film is to crosslink the matrix. This can be accomplished chemically (peroxides, sulfur vulcanization) (Gagnon *et al.*, 1994a; Gagnon *et al.*, 1994b) or physically (radiation) (De Koning *et al.*, 1994). While chemical methods are effective, they introduce unwanted substances into the system. In contrast, the radiation of polymeric materials generally results in three-dimensional network structures with improved tensile properties without the addition or formation of con-

taminants in the matrix. Gamma-irradiation was chosen in this study because of its high penetration power and ability to undergo exponential attenuation in the irradiated material. PHA films produced from coconut oil (PHA-C; low olefin concentration), tallow (PHA-T; intermediate olefin concentration), and soybean oil (PHA-S; high olefin concentration) were irradiated to determine the effects of olefin content on radiation-induced crosslink efficiency and tensile properties.

Materials and methods

Materials

All simple salts and coconut oil were obtained from Sigma Chemical Company, tallow was obtained from Miniatur Inc. (Chicago, IL) and soybean oil was from the supermarket under the trade name Wesson. All organic solvents were HPLC grade and purchased from Burdick and Jackson (Muskegon, MI).

Strain information and polymer synthesis

Pseudomonas resinovorans NRRL B-2649 was obtained from the NCAUR, ARS, United States Department of Agriculture, Peoria IL, and used as the producer strain in all fermentations. Stock cultures were prepared as described previously (Ashby and Foglia, 1998) and stored at -70°C until use as the inocula for the fermentations.

Fermentations were conducted in 500 mL volumes under batch culture conditions in Medium E (pH 7.0) (for medium composition see Lageveen *et al.*, 1988). Each TAG (coconut oil, tallow, and soybean oil) was heated at 60°C for 15 minutes and added to sterile Medium E at a concentration of 1 g/L. The flasks were inoculated with a 0.1% (v/v) inoculum from a thawed cryovial. Bacterial growth and polymer production were carried out at 30°C with shaking at 250 rpm for 72 h in an orbital shaker. At 72 hours the cultures were pelleted by centrifugation (8000 × g, 20 min., 4°C) and washed twice in deionized water. Unused fats (solids) were removed from the cultures prior to centrifugation by filtration through cheesecloth. Unused oils concentrated at the air/liquid interface upon centrifugation and were removed either with the supernatant or by wiping the walls of the centrifuge bottles with paper towels. The cell pellets were then lyophilized (~24 h) to a constant weight.

PHA purification and isolation

Supercritical fluid extraction (SFE) was used to remove residual TAG materials prior to PHA isolation. The dried bacterial cells (5 g per extraction) were packed tightly into a 24 mL stainless steel extraction vessel (rated at 680 atm). The extraction vessel was placed in the SFE apparatus oven (Applied Separations, Allentown, PA). The oven was adjusted to 60°C and the restrictor valve temperature was set at 100°C. When the vessel temperature reached 60°C, carbon dioxide was allowed to flow through the extraction vessel at 1.5 L/min. at 340 atm (1 h), 476 atm (1 h), and 612 atm (1 h), consecutively. The bacterial cells were removed from the extraction vessel and the PHA isolated following the procedure of Shi *et al.* (1996).

Instrumental procedures

Each *mcl*-PHA was characterized with respect to repeat unit composition, molar mass, and thermal properties prior to film formation. PHA repeat unit compositions were determined by gas chromatography (GC) and GC/mass spectrometry (GC/MS) of the silylated and non-silylated 3-hydroxymethyl esters (3-HMEs) using conditions reported previously (Brandl *et al.*, 1998). The methyl esters were silylated by reacting 10 µL of each sample with 250 µL *N,O*-bis(trimethylsilyl)-trifluoroacetimide (BSTFA) and 200 µL pyridine. The mixtures were heated at 70°C for 30 minutes and allowed to cool to room temperature. Finally, 150 µL of hexane was added to each sample and the samples analyzed by GC/MS.

Molar mass averages were determined by gel permeation chromatography (GPC) after the procedure of Cromwick *et al.* (1996). Thermal properties and the carbon nuclear magnetic resonance (¹³C-NMR) spectra of each *mcl*-PHA

were obtained as described elsewhere (Ashby and Foglia, 1998).

Film preparation and irradiation

Films were cast from solutions of PHA-T, PHA-C, or PHA-S. Solutions were prepared by dissolving 1.5 g of purified PHA in 15 mL of chloroform. Films were cast in glass petri dishes (100 mm × 15 mm), the solvent evaporated in a nitrogen atmosphere, and the films dried under vacuum at 20°C for 7 h. The resulting films (approximately 0.1 mm thick) were stored desiccated in the dark under nitrogen prior to irradiation. The films in the glass petri dishes were irradiated with 50 kGy of radiation (energy was based on an 8.5 h exposure) using a cesium-137 source at 20°C in a nitrogen atmosphere. The irradiator and dosimetry were described previously (Shieh *et al.*, 1985).

Sol/gel analysis

Sol/gel tests were performed on non-irradiated and irradiated films. A 60–100 mg section of each film was accurately weighed, placed in a glass vial containing 3 mL of chloroform at room temperature for 24 h, and occasionally agitated. The swollen films were retrieved by filtering the chloroform through a pre-weighed 0.45 µm nylon filter membrane that had been dried overnight at 60°C. The filters (containing the gel fractions) were then placed in a 60°C oven overnight and reweighed.

Tensile property measurement

Tensile properties of the PHA films were measured at 23°C and 50% relative humidity with a gauge length of 25 mm. An Instron tensile testing machine, model 1122 (Canton, MA), was used with a cross-head speed of 50 mm/min. Measurements included tensile strength, elongation to break and Young's modulus. Tensile strength and elongation to break were defined as the ultimate stress and strain, respectively. Young's modulus is a physical quantity representing the stiffness of a material. It was determined by measuring the slope of a line tangent to the initial stress-strain curve.

Results and discussion

We previously reported that *P. resinovorans* produced *mcl*-PHAs that were compositionally distinct based on the fatty acid make-up of the fat or oil substrate (Ashby and Foglia, 1998). Specifically, the side-chain alkyl residues of the PHA polymers contained double bonds that varied with the original unsaturation of the TAG substrate. In general, the greater the percent residual double bonds, the lower the crystallinity of the PHA. In this report, *mcl*-PHA polymers were produced from tallow, coconut oil, and soybean oil so that the effects of radiation on polymer film

properties could be measured and compared to non-irradiated films. In a single stage fermentation the crude cell yields ranged from 2.4 g/L (tallow) to 4.1 g/L (coconut or soybean oil) and resulted in *mcl*-PHA yields equivalent to 43% (tallow) to 60% (coconut or soybean oil) of the cell dry weight (volumetric yields = 1.0 g/L for tallow, and 2.5 g/L for coconut and soybean oil). The ability of *P. resinovorans* to grow and produce equal cell masses and polymer yields on coconut oil (highly saturated TAG) and soybean oil (highly unsaturated TAG) indicated that the concentration of *cis* double bonds had little effect on substrate metabolism. This suggested that the discrepancy between cell growth and PHA yields on tallow *vs* coconut or soybean oil was primarily due to the physical state of the substrate at 30°C (incubation temperature) rather than its fatty acid composition. The titer (melting range) of coconut oil and soybean oil is each less than 25°C. This allows these oils to be easily dispersed in the culture media by simple agitation at 30°C. In contrast, tallow has a titer of greater than 40°C, substantially higher than the incubation temperature. The result was that the bulk of the tallow remained a solid throughout the fermentation, which limited bacterial growth and polymer yield. In addition to TAGs, animal fats and vegetable oils contain small amounts of sterols and tocopherols. Utilization of these compounds for cell growth and polymer production cannot be eliminated. High cell and polymer yields, along with polymer composition, however, suggest that TAGs were the primary carbon sources used by the bacterium.

It is well known that unsaturation enhances the ability of a material to oxidize and form chemical crosslinks (Mercx *et al.*, 1996). For this reason, *mcl*-PHA polymers were sought that contained a range of side-chain double bonds. The compositions of the *mcl*-PHA polymers were determined by GC and GC/MS of the 3-HMEs (Table 1).

The olefin content was calculated, based on the total number of carbons in each polymer, from the GC composi-

tional data and confirmed by ¹³C-NMR. Coconut oil, composed of approximately 95% saturated fatty acids, gave an *mcl*-PHA polymer with side-chains containing 0.3 mole% olefinic carbons. Tallow, whose main constituent is oleic acid (~37%), gave an *mcl*-PHA with 1.7 mole% olefinic carbons. Soybean oil contained the highest amounts of unsaturated fatty acids (21% oleic acid, 57% linoleic acid, 8% linolenic acid). Accordingly, soybean oil gave an *mcl*-PHA polymer containing 5.1 mole% olefinic carbons.

As the olefin concentration increased, the polymers became more amorphous. This was verified by thermal analysis of each polymer (Table 2). PHA-C and PHA-T were elastomeric at room temperature. PHA-C had the highest glass transition temperature (T_g), melting temperature (T_m), and enthalpy of fusion (ΔH_m) of the three polymers. While it seems likely that these increases were primarily due to a more ordered packing arrangement of the saturated side-chains, the increased molar mass of PHA-C also may have helped to increase the T_g and T_m of PHA-C. At the other extreme, PHA-S had a T_g of -47°C; T_m and ΔH_m , however, were not measurable. This indicated that PHA-S was completely amorphous and, in fact, was liquid-like at room temperature. This was probably due to the smaller molar mass and higher concentration of double bonds in the side-chains, which resulted in a lack of packing uniformity in the polymer.

In addition to composition, molar mass also influences the tensile properties of a polymer. The molar masses of the PHA polymers are seen in Table 2. The decrease in the number average molar masses (M_n) from PHA-C to PHA-S suggested that an increased concentration of unsaturated fatty acids in the substrate interrupted the polymerization efficiency of the system. Because equal polymer yields were obtained from both coconut oil and soybean oil, it seems likely that the presence of unsaturated fatty acids, while not inhibiting polymerization, caused a more efficient termination of polymerization and resulted in a larger

Table 1 The repeat unit composition of triacylglycerol-based *mcl*-PHA isolates.

Isolate	3-hydroxymethyl ester (area %) ^a									
	C _{4:0}	C _{6:0}	C _{8:0}	C _{10:0}	C _{12:0}	C _{12:1}	C _{14:0}	C _{14:1}	C _{14:2}	C _{14:3}
PHA-C	tr ^b	7	33	40	16	1	3	tr		
PHA-T	tr	3	15	46	17	4	6	9		
PHA-S	tr	4	18	32	8	14	4	9	10	tr

^a Average relative area percent (n = 5) as determined by GC of the 3-hydroxymethyl esters obtained by acid hydrolysis and methylation of each *mcl*-PHA polymer.

^b tr (trace) = less than one percent.

Table 2 Molar mass and thermal^a properties of triacylglycerol-based *mcl*-PHA isolates.

Isolate	M_n ($\times 10^3$)	M_w ($\times 10^3$)	M_w/M_n	T_g (°C)	T_m (°C)	ΔH_m (J/g)
PHA-C	133	449	3.38	-40	44	15.4
PHA-T	93	269	2.89	-46	42	11.7
PHA-S	57	121	2.13	-47	*	*

^a All thermal analyses were performed by differential scanning calorimetry (DSC).

* PHA-S was amorphous and showed no melting transition.

number of smaller molar mass PHA-S chains. In addition, previous reports have shown that 3-hydroxyacyl-CoA intermediates from the β -oxidation of fatty acids serve as precursors for PHA biosynthesis in *P. putida* (Eggink *et al.*, 1993; De Waard *et al.*, 1993; Lageveen *et al.*, 1988). Unsaturated free fatty acids require additional isomerase and epimerase enzymes for oxidation to produce the CoA intermediates required for polymerization. The increased energy requirement involved in the production of two additional enzymes may slow the polymerization process and result in polymers with smaller relative molar masses. Whatever the predominant explanation, it may be that both chemistries exist, resulting in a molar mass reduction in *mcl*-PHAs containing higher concentrations of unsaturated side-chains.

Contaminants, such as residual TAG substrate, may change the tensile properties of the polymer films. To obtain accurate tensile measurements, each *mcl*-PHA polymer was purified to remove residual TAG substrate prior to film formation. In a previous report, we showed that the solubility of PHA-T was very similar to tallow, making purification by differential solubilities in organic solvents difficult (Ashby *et al.*, 1998). SFE in carbon dioxide provided a means to purify the *mcl*-PHAs without using large volumes of organic solvent. We found that TAGs are soluble in supercritical CO₂, whereas the intracellular *mcl*-PHA polymers are not. Hence, by extracting the dried bacterial cells the residual fat or oil substrate material was removed without polymer loss. GC and GC/MS of PHA-T showed the presence of fatty acid methyl esters (FAMES) from residual TAG contaminants. After extraction in supercritical CO₂, GC showed that the residual FAMES from the TAG substrate were absent while the 3-HMEs remained. This verified the purification efficiency of the SFE procedure and helped limit the total volume of organic solvent needed to isolate the pure polymers.

Radiation effects

Radiation affects the properties of polymeric materials according to the type and source of radiation, the nature of the polymer structure, and the mechanism of reaction. It has been well established that crosslinking of a polymeric material requires the presence of functional groups (Mercx *et al.*, 1996). Olefinic groups are used frequently to this end since they can be crosslinked by both chemical (Gagnon *et al.*, 1994a; Gagnon *et al.*, 1994b) and radiation methods (De Koning *et al.*, 1994). As seen from the *mcl*-PHA compositions (Table 1), many of the double bonds from the TAG substrate were preserved by the bacterial PHA polymerization process. These groups provide the functionality necessary for crosslinking. Gamma-irradiation

Table 3 Tensile properties of irradiated and non-irradiated *mcl*-PHA polymer films.

Isolate	Radiation Dose (kGy) ^a	Tensile Strength (MPa)	% Elongation	Young's Modulus (MPa) ^b
PHA-C	0	2.5	242	2.0
	50	5.1	360	2.6
PHA-T	0	3.0	320	1.7
	50	4.9	360	3.0
PHA-S	0	*	*	*
	50	0.7	25	3.1

^a Radiation source was cesium-137.

^b Young's modulus was determined by measuring the slope of a line tangent to the initial stress-strain curve.

* In the absence of radiation PHA-S was amorphous and resulted in a non-coherent film.

of a polymeric material generates radicals that lead to crosslink formation. However, these radicals also cause chain scission, the extent of which depends on the chemical composition of the material. Formally, the two processes, *i.e.*, crosslinking and chain scission, occur simultaneously, making it necessary to achieve some balance between the two processes to enhance the tensile properties of each material.

In the present study, *mcl*-PHAs were γ -irradiated and evaluated with respect to changes in tensile properties. After irradiation, each polymer film was subjected to sol/gel analysis to determine the extent of crosslinking using non-irradiated films as controls. For non-irradiated films, the gel fraction amounted to between 1.0% (w/w) and 1.4% (w/w) of the total film weight tested. This indicated that prior to irradiation the films were relatively crosslink-free. After irradiation, the gel fractions increased to 8% (w/w), 60% (w/w), and 91% (w/w) for PHA-C, PHA-T, and PHA-S, respectively. Accordingly, the extent of crosslinking was proportional to the number of olefinic groups present in each *mcl*-PHA polymer side-chain. Tensile properties were measured immediately after irradiation to limit auto-oxidation. In each case, the tensile properties were enhanced by irradiation (Table 3).

The most interesting result occurred with PHA-S films. While the tensile properties of the PHA-T and PHA-C films increased, the PHA-S films were stiffened from a liquid-like texture to a solid film. The tensile strength and percent elongation of the irradiated PHA-S film were 0.7 MPa and 25%, respectively, showing it to be a weaker film than the crosslinked PHA-T and PHA-C films. Young's modulus, which is a measure of the stiffness of a material, also increased in each polymer film after irradiation. Inter-

Table 4 Sol fraction^a molar masses of the irradiated *mcl*-PHA polymer films.

Isolate (film)	Radiation Dose (kGy)	Molar Mass (sol fraction)		
		$M_n (\times 10^3)$	$M_w (\times 10^3)$	M_w/M_n
PHA-C	50	59	205	3.47
PHA-T	50	48	194	4.04
PHA-S	50	33	102	3.09

^a Sol fractions were obtained from the chloroform soluble portions of the sol/gel analysis after film irradiation.

estingly, PHA-S had the highest modulus after irradiation. These results indicated that irradiation had the greatest effect on the PHA-S films, which is directly related to the higher olefin concentration.

Gel permeation chromatography (GPC) of the soluble (sol) fractions from the sol/gel analyses of the irradiated polymers (Table 4) showed that the molar masses were smaller and more polydisperse than non-irradiated controls (Table 2).

This indicated that, in addition to crosslinking, chain scission was also occurring during the radiation process. The formation of small quantities of large molar mass polymers increased polydispersity (M_w/M_n) (see Figure 1). Specifically, the total amount of large molar mass fraction material varied from 3% (PHA-S) to 18% (PHA-C) with average M_n values well above 6.0×10^6 g/mol. Because irradiation results in a balance between crosslinking and

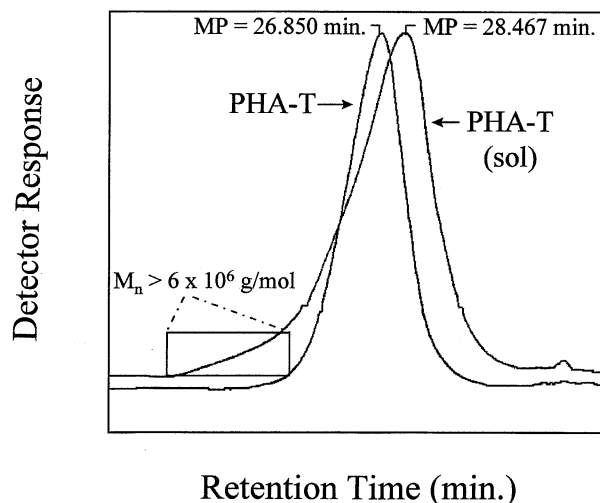


Figure 1 Gel permeation chromatogram of pure PHA-T and the soluble (sol) fraction of radiation crosslinked PHA-T derived from sol/gel analysis. "MP" = the mid-point retention time (min.) for each peak.

chain scission, it is entirely possible that the large molar mass fraction was the result of the radiation-induced formation of microgels that are small enough to pass through a $0.45 \mu\text{m}$ filter prior to GPC analysis. PHA-C is composed of the fewest olefinic carbons. This results in a limited number of crosslink sites compared to chain scission sites. Because of this, a larger concentration of microgels may be produced from PHA-C when compared to PHA-S during irradiation. This combination of microgel formation and chain scission increased the polydispersities for the sol fractions of the irradiated films.

In conclusion, the data show that γ -irradiation enhanced the tensile properties of each *mcl*-PHA polymer film despite the fact that, in addition to crosslinking, each film endured simultaneous chain scission. In each case, the crosslink density was proportional to the side-chain olefin concentration. PHA-S was highly crosslinked after irradiation as evidenced by the large gel fraction upon sol/gel analysis. This was due to the high concentration of olefinic groups in the PHA-S side-chains. PHA-T and PHA-C both showed some crosslinking after radiation exposure. However, the crosslinking of these two polymers was not as dramatic as PHA-S because of the reduced number of double bonds in their side-chains. These results indicate that the extent of unsaturation in an *mcl*-PHA side-chain plays an important role in polymer crosslinking. While crosslinking increased the tensile properties of each *mcl*-PHA polymer film, chain scission probably retards the magnitude of this increase by depolymerizing the polymer films to some extent. It would be ideal if radiation-induced crosslinking could be achieved without chain scission. This may be possible by increasing the size of the side-chains, increasing the side-chain olefin content, or incorporating hetero atoms, such as oxygen, into the film matrix that are more easily ionizable.

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